

EXCRETION OF NITROGE- NOUS SUBSTANCES IN THE PROTOZOA

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EXCRETION OF NITROGENOUS SUBSTANCES IN PROTOZOA¹

(Four figures)

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I. NATURE OF NITROGENOUS END-PRODUCTS OF METABOLISM IN PROTOZOA

THE nitrogenous end-products of metabolism in organisms vary in their nature according to the type of organism and its physiological condition. For example, in man the bulk of the nitrogen is eliminated as urea, and considerably less as uric acid, amino acids, and other nitrogenous compounds; while in birds and reptiles the bulk of the nitrogen is eliminated as uric acid. The question then arises: In what form is nitrogen excreted by Protozoa—as ammonia, urea, uric acid, or other substances, or a combination of these?

Griffiths (1888) concludes that the contractile vacuoles of certain Protozoa perform the function of a kidney and that their secretions are “capable of yielding microscopic crystals of uric acid.” In his experiments he used *Amoeba sphaerococcus* (Haeckel), *Vorticella* sp., and *Paramecium bursaria*. A number of the organisms were subjected to the murexide test. The development of reddish-purple color indicated the presence of uric acid. In describing these experiments, Griffiths says (p. 132): “After the addition of alcohol minute flakes could be distinctly seen floating in the fluid of the vacuoles. Bearing in mind the murexide reaction, there is every reason to believe that these flakes are nothing more or less than minute crystals of uric acid.” These experiments were repeated many times, generally with positive results, indicating the presence

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of uric acid. At times, however, the vacuole was found not to contain the slightest trace of uric acid.

Howland (1924) repeated these experiments, using several specimens of *Centropyxis* and *Amoeba verrucosa*. No crystals of uric acid were found in the distended vacuoles after the addition of alcohol; nor was the characteristic murexide reaction for uric acid obtained after the addition of ammonia, either in the vacuoles or in the culture medium immediately surrounding the organism. These experiments were conducted over a period of 5 weeks, always with negative results. However, by making use of the Benedict blood-filtrate test, Howland demonstrated the presence of uric acid in cultures of *Amoeba* and *Paramecium*. In this test the presence of uric acid is evidenced by the development of blue color, the intensity of the color increasing with a corresponding increase in concentration of uric acid. In Howland's experiments the depth of the color varied with the age of the cultures, the older ones giving a deeper color. From this she concluded that these organisms excrete uric acid.

Adolph (1922) concludes that "either waste-products are not deleterious through their retarding of metabolism, or urea is not an excretory product in *Phagocata* and *Paramecium*."

These observations appear to offer the only direct evidence concerning the nature of nitrogenous end-products in Protozoa. Since the evidence is so limited, and since that which exists is not wholly in agreement, the question cannot be considered definitely answered.

MATERIAL AND METHODS

In an effort to answer this question the following experiments were designed. A large number of organisms—*Paramecia*, *Spirostoma*, or *Didinia*—were thoroughly washed, placed in modified Ringer's solution,¹ allowed to remain for a time, and then removed by filtration. The filtrate was tested for various nitrogenous substances according to methods described below.

The *Paramecia* were washed as follows: Culture fluid containing the organisms was poured into a long-necked bottle of approximately 1 liter capacity until it was filled to within about 5 cm. of the top. Then tap water was added carefully to avoid mixing, until the bottle was entirely full. Within 5 or 10 minutes under these conditions

¹ CaCl₂ 2.22 grams, KCl 0.178 grams, NaCl 2.24 grams, water to make 30 liters.

the *Paramecia* usually aggregate in great numbers near the surface of the water. When they had thus collected at the top of the bottle, the surface water containing the *Paramecia* was removed with a pipette, and more tap-water added. This process was repeated, usually three or four times, until most of the organisms had been removed from the culture fluid. The bottle was now emptied, and the water containing the *Paramecia* put into it; then it was filled with tap-water and left until the animals came to the surface, after which they were removed as before. The *Paramecia* were thus washed in tap-water three or four times, after which they were usually found to be free from all heavy débris and large bacterial masses. Smaller organisms which were removed with the *Paramecia* were separated from the *Paramecia* by further washing on filter paper. The paper was about 20 cm. in diameter, and contained pores small enough to retain the *Paramecia* but large enough to allow the smaller organisms to pass through. About 2 liters of water, more if necessary, were used for this part of the washing process. The last part of this wash-water was the modified Ringer's solution previously mentioned. This solution was used so that a uniform medium of known composition might be used for all the various experiments. The animals seem to live very satisfactorily in it. The *Paramecia* were then transferred to a clean beaker, and the number per cubic centimeter ascertained by counting those in several measured volumes and averaging the results. In the experiments on *Paramecium* the numbers range from 1,000 to 3,500 per cubic centimeter. That the animals were sufficiently washed is evidenced by the fact that tests for various nitrogenous substances made immediately after the completion of this procedure were invariably negative.

Neither *Spirostomum* nor *Didinium* aggregates in the upper part of the solution as readily as *Paramecium*, so the washing process had to be limited to such as could be accomplished on filter paper. Since negative results were obtained in tests for the various nitrogenous substances, which were made immediately after washing was completed, it is concluded that washing in this manner was sufficient. The numbers per cubic centimeter were ascertained as for *Paramecium*. There were from 300 to 1,000 *Spirostoma* and from 160 to 500 *Didinia*.

After the organisms had been washed as described above, they

were allowed to remain in the modified Ringer's solution for various periods of time. At intervals samples of the solution were removed, filtered, and the filtrate subjected to chemical tests for ammonia, urea, uric acid, creatin, and creatinin as follows:

Ammonia by direct Nesslerization.—In the presence of ammonia Nessler's reagent gives a distinctive reddish-brown color, which varies in intensity according to the concentration of ammonia. By comparing the intensity of color produced in a solution of ammonia of unknown concentration to that produced in a solution of ammonia of known concentration this test may be used as a quantitative indicator.

Urea by hydrolysis to ammonium carbonate by means of urease,¹ and subsequent determination of ammonia thus produced by Nesslerization.—In so far as it is known, urease is specific in its action on urea.

Uric acid by Benedict blood-filtrate test.—Five cc. of the unknown solution are treated with 1 cc. of a 5 per cent solution of sodium cyanid in order to intensify the color produced by a modified phosphotungstic acid reagent. The chief modification by Benedict of the original Folin method is the introduction of arsenic pentoxid to form an arsenophosphotungstic acid reagent, which increases its specificity for uric acid.

Creatin by converting it to creatinin by hydrolysis, and testing for creatinin.—To one volume of the unknown add an equal volume of a 10 per cent solution of hydrochloric acid. The mixture is then placed in an autoclave for 10 minutes at a temperature of 115° C.

Creatinin by Jaffe's test.—To 5 cc. of the unknown solution add 1 cc. of a saturated solution of picric acid and 5 drops of a 10 per cent solution of sodium hydroxide. If the unknown contains creatinin, the solution gradually becomes reddish owing to the formation of creatinin picrate.

Creatinin by Weyl's test.—To 5 cc. of the unknown solution add 5 drops of a 5 per cent solution of sodium nitroferrocyanid (nitroprussid) and a few drops of a 5 per cent solution of sodium hydroxide.

¹ Urease-Dunning, prepared by Hynson, Westcott, and Dunning, Baltimore, Maryland.

The appearance of a permanganate color indicates the presence of creatinin.

RESULTS

The results of the analyses of the filtrates are presented in Table I. This table indicates that *Paramecium* excretes urea, but no ammonia, uric acid, creatin, or creatinin. Solutions in which *Paramecia* were allowed to remain for several hours gave a decidedly positive test for ammonia with Nessler's reagent after hydrolysis with urease. No evidence of ammonia was obtained before hydrolysis. Urea, then, must have been hydrolyzed with the subsequent production of the ammonia found after the addition of urease.

TABLE I

NITROGENOUS EXCRETORY PRODUCTS OF *Paramecium*, *Spirostomum*, AND *Didinium*

Organism	Ammonia	Urea	Uric Acid	Creatin	Creatinin
<i>Paramecium</i>	none	some	none	none	none
<i>Spirostomum</i>	none	some	none	none	none
<i>Didinium</i>	some	none	trace?	none	none

In tests in which the *Paramecia* remained in the solution comparatively long (12 hours, or more), a positive indication of ammonia was often found before the addition of urease. This ammonia undoubtedly arose from hydrolysis of urea by bacteria which had accumulated in the solution. That the bacteria which are found in *Paramecium* cultures hydrolyze urea was demonstrated in the following manner. A dilute solution of urea was inoculated with culture fluid. After the solution had been allowed to stand for several hours, it gave a positive indication of ammonia with Nessler's reagent. Furthermore, the length of time required for ammonia to make its appearance was not dependent upon the number of *Paramecia* present. If excretion by the *Paramecia* had been the source of this ammonia, a direct relation between these two factors should have been evident. Ammonia appeared only after the experiment had been in progress a considerable length of time. The methods for washing the *Paramecia* were such that a minimum of 12 hours was necessary for the bacteria which had not been removed to multiply to such an extent as to hydrolyze enough urea to give a positive reaction with Nessler's reagent.

Essentially the same results were obtained with *Spirostomum* as with *Paramecium*. Urea was the only nitrogenous substance found.

In tests with *Didinium*, however, a very definite indication of ammonia was obtained without the use of urease; and the depth of color developed after the addition of this enzyme did not appear greater than that produced in another portion of the same sample to which it had not been added. This was taken to indicate that no urea is excreted by this form. If the organisms were allowed to remain in the solution for as long as 24 hours, a trace of uric acid was sometimes found, but the quantity was so slight as to make impossible an estimate with any considerable degree of accuracy. No evidence whatever was obtained of the presence of creatin or creatinin.

As previously stated, the tests for uric acid in experiments with *Paramecium* and *Spirostomum* were negative at all times. In one experiment 2,800 *Paramecia* per cubic centimeter of solution were allowed to remain in the modified Ringer's solution for 72 hours; in another, 2,500 per cubic centimeter for 6 days. In neither of these experiments, and in none of those in which the length of time was shorter, was any indication of uric acid obtained. These results were unexpected in view of the fact that Griffiths claimed to have seen crystals of uric acid in the vacuoles of *Amoebae*, and Howland gave conclusive evidence concerning the presence of uric acid in cultures of Protozoa. Griffiths' experiments, however, were not confirmed by Howland although she followed his technique specifically. But the presence of uric acid in mass cultures has been repeatedly confirmed by the author. The question then arises as to the source of this substance.

The author demonstrated that uric acid is not eliminated by *Paramecia* or *Spirostoma* kept in modified Ringer's solution or tap-water, but he almost invariably found it in samples taken from ordinary cultures. Furthermore, the quantity, according to Howland, increases with the age of these cultures. If all these statements are valid, then the uric acid found in the culture fluid must have originated in substances from which the fluid was made. Various substances commonly used in the preparation of culture media—hay, wheat, barley, rye, oats, malted milk, beef extract, blood fibrin, and blood albumen—were therefore tested for uric acid. Without

a single exception, a cold aqueous solution or extract of each of these substances yielded with the Benedict blood-filtrate test unmistakable evidence of uric. The fact that Howland found that old cultures gave more strongly positive tests may be explained in the following manner. As the cultures become older, more and more uric acid dissolves from the material used in the preparation of the medium. The solubility of uric acid in cold water is comparatively slight, which would tend to produce a slow rate of diffusion from the substance which contains it, and a corresponding increase in concentration with the passage of time. Or, another conceivable source of uric acid which would give an increased concentration with the passage of time is the decomposition of proteins in the culture medium.

II. RATE OF EXCRETION OF NITROGENOUS END-PRODUCTS OF METABOLISM IN PROTOZOA

In 24 hours the average adult human male excretes about 15 grams of nitrogen (Morse, 1927, p. 696). This amount varies greatly according to the physiological condition—hunger, nature of diet, etc. For the fourteenth day of starvation the amount eliminated is only about half what it is in well-fed individuals; i.e., 7.7 grams (Morse, 1927, p. 568). So far as the author has been able to ascertain, there is no evidence concerning the rate of excretion in Protozoa, or concerning the effects of starvation and satiety on the rate of excretion. With these facts in mind experiments were designed which have a direct bearing on these questions.

MATERIAL AND METHODS

In these experiments the three forms previously mentioned—*Paramecium*, *Spirostomum*, and *Didinium*—were used. The rate of excretion of nitrogenous substances was ascertained in the following manner: The organisms were washed according to methods previously described, and put into modified Ringer's solution. At appropriate time intervals samples of the fluid were removed and the quantities of nitrogenous substances contained therein measured by methods described in a preceding section. In these experiments various numbers of organisms were used, but the results were calculated on the basis of the following numbers per cubic centimeter for

the different species: *Paramecium*, 2,500; *Spirostomum*, 500; and *Didinium*, 200.

RESULTS

The results obtained in these experiments are presented in Tables II, III, and IV and in Figures 1, 2, 3, and 4.

Paramecium.—By referring to Table II and Figure 1, it will be seen that the amounts of ammonia produced by hydrolysis of the

TABLE II
RATE OF EXCRETION OF UREA IN *Paramecium**

Exp. No.	No. of Animals per Cc.	Time of Washing (Hr.)	AMMONIA: PARTS PER 1,000,000 BY WEIGHT FOR 2,500 ANIMALS PER Cc. EXCRETED IN—								
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.	12 Hr.
3.....	1,000	1	0.87	1.20	1.20		1.20				
4.....	2,500	$\frac{1}{2}$	0.82	1.17	1.17	1.17				1.17	
7.....	2,000	$\frac{1}{2}$	0.90	0.98	1.10	1.15		1.25			
8.....	2,200	1	0.77	0.90			0.90	17 hr. 0.90			
Average			0.84	1.06	1.16	1.16	1.05				
1.....	2,500	1				1.80				1.90	2.00
2.....	1,000	$\frac{1}{2}$		1.65		1.95				1.95	2.20
5.....	2,500	1	0.65	1.15	1.57	1.75				1.75	
6.....	1,500	$\frac{1}{2}$		1.02			1.82			2.00 (17 hr. 2.00)	
Average			0.65	1.27	1.57	1.83	1.82			1.90	

* The amount of urea excreted by 2,500 animals per cubic centimeter of solution in 1-12 hours is given in parts of ammonia by weight per 1,000,000 of solution after hydrolysis. To convert parts of ammonia to parts of urea, multiply figures given by 1.76. Note that the rate of excretion is nearly zero after 2 hours.

urea present at successive periods, when plotted as ordinates against time as abscissae, form curves of a fairly uniform type; and that these curves rise at a nearly constant rate to a point indicating a concentration of urea sufficient to produce 0.90-1.25 parts by weight of ammonia per 1,000,000 of solution after hydrolysis, and then become practically horizontal. This indicates that excretion continued at a uniform rate during the first 1 or $1\frac{1}{2}$ hours, and then ceased entirely or fell to a very low rate. Essentially the same thing

is shown in Table II and Figure 2. The curves in this figure indicate, however, that excretion continued until sufficient urea had accumulated to produce, after hydrolysis with urease, a concentration of

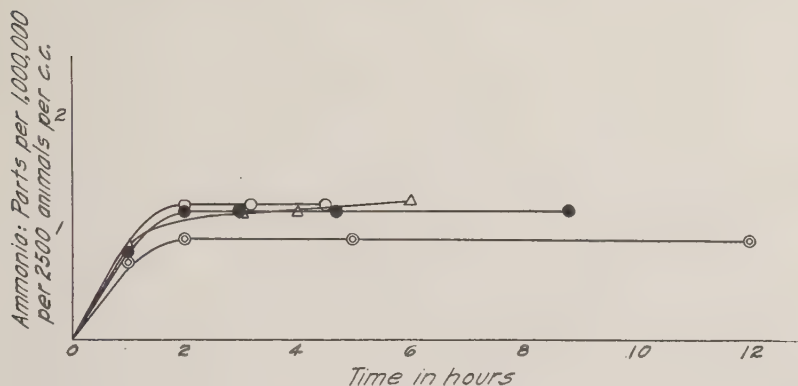


FIG. 1.—*Paramecium*: concentration of urea in surrounding medium at successive periods of time as indicated by the amount of ammonia produced by hydrolysis. ○, Exp. 3; ●, Exp. 4; △, Exp. 7; ⊙, Exp. 8.

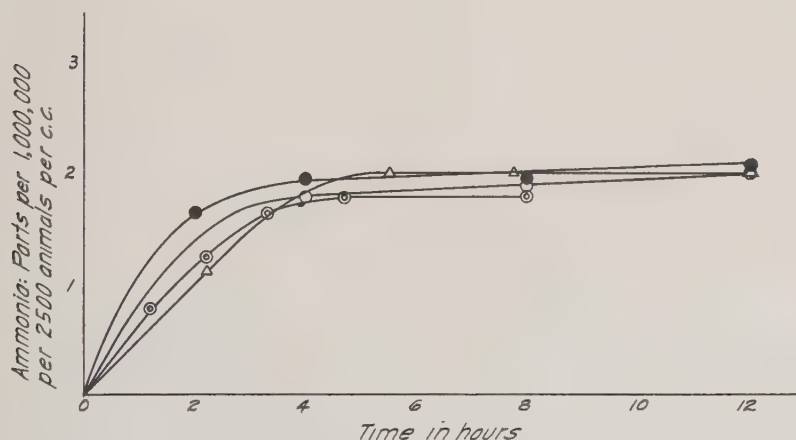


FIG. 2.—*Paramecium*: concentration of urea in surrounding medium at successive periods of time as indicated by the amount of ammonia produced by hydrolysis. Amount of ammonia produced by hydrolysis at successive periods of time. ○, Exp. 1; △, Exp. 2; ⊙, Exp. 5; ●, Exp. 6.

ammonia of about 1.75–3.10 parts by weight per 1,000,000 of solution, requiring from about $2\frac{1}{2}$ –4 hours before the rate of excretion fell to a minimum.

From the slope of the curve it is possible to compute the rate of excretion at any period. Thus, during the first hour represented in Figure 1, and the first 2 hours in Figure 2, the rate of excretion was approximately 1.40 parts of urea per 1,000,000 by weight per hour for 2,500 animals per cubic centimeter.

The question now arises as to what factors are correlated with the rapid decrease in the rate of excretion observed. As previously stated, in man the rate of excretion is specifically correlated with the amount of food available. Observations made on the relative abundance of food vacuoles in *Paramecium* indicate that this also

TABLE III
RATE OF EXCRETION IN *Spirostomum**

Exp. No.	No. of ANIMALS PER CC.	TIME OF WASHING (MIN.)	AMMONIA: PARTS PER 1,000,000 BY WEIGHT FOR 500 ANIMALS AFTER—							
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.
1	275	20		1.70		2.55				2.55
2	425	20		2.00		3.00				3.00
3	350	15	0.80	1.05	1.87		2.85			2.85
4	400	15	1.00	1.17		1.90				1.90
Average			0.90	1.48	1.87	2.48	2.85			2.58

* The amount of urea excreted by 500 animals per cubic centimeter of solution in 1-8 hours is given in parts of ammonia by weight per 1,000,000 of solution after hydrolysis. To convert parts of ammonia to parts of urea, multiply figures given by 1.76.

obtains for Protozoa. In these observations it was found that the food vacuoles were abundant at the beginning of the experiments but that after starvation for several hours they were entirely or almost entirely lacking. Coincident with the disappearance of the food vacuoles was the change in direction of the curve, indicating a decrease and finally the cessation of excretion.

Spirostomum.—Essentially the same type of phenomena were observed in the experiments on *Spirostomum* as in *Paramecium*. The initial rate of excretion was found to be approximately 1.25 parts of urea by weight per 1,000,000 of solution per hour for 500 animals per cubic centimeter. This rate of excretion continued for 3-4 hours, after which it decreased to a very low figure or to zero (Table III and Fig. 3).

Didinium.—Experiments on *Didinium* yielded more direct evidence concerning the effects of starvation and satiety on the rate of excretion than was obtained in the observations on *Paramecium*. As previously stated, *Didinium* excretes ammonia, whereas *Paramecium* and *Spirostomum* excrete urea. Four tests were made to ascertain the rate of excretion of ammonia in *Didinium* according

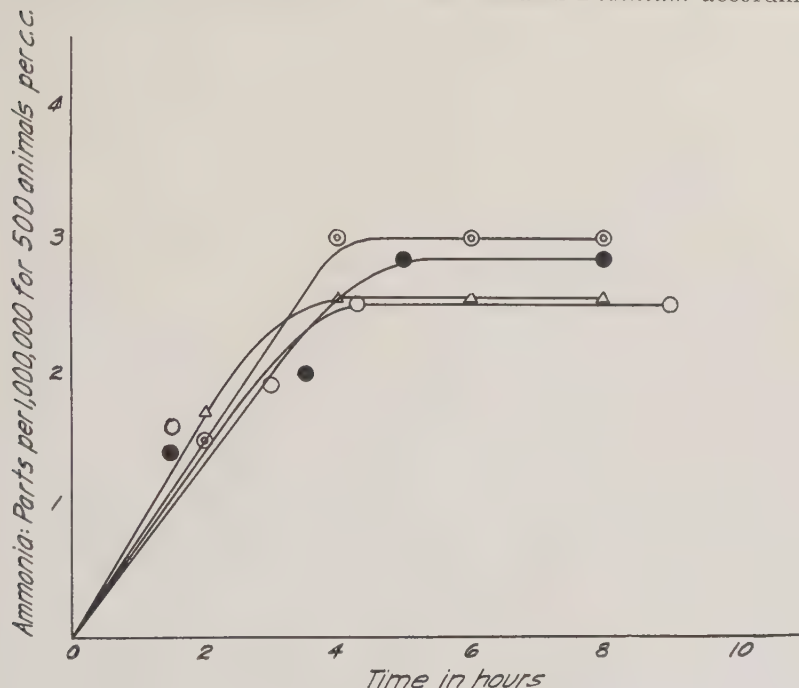


FIG. 3.—*Spirostomum*: concentration of urea in surrounding medium at successive periods of time as indicated by the amount of ammonia produced by hydrolysis. Amount of ammonia produced by hydrolysis at successive periods of time. Δ , Exp. 1; $\circ$, Exp. 2; $\bullet$, Exp. 3; $\circ$, Exp. 4.

to methods previously described. In two of the tests only a few *Paramecia* were added as food to the fluid containing the *Didinia*. From these experiments were obtained curves 2 and 3 (fig. 4). In the other two enough *Paramecia* were added to provide sufficient food for the duration of the experiments. These *Paramecia* had been starved for a period of 12 hours or longer, so the amount of nitrogenous substances excreted by them was insignificant. The results of these four experiments are presented in Table IV and in Figure 4.

By comparing curves 1 and 4 with curves 2 and 3 in Figure 4, it will at once be seen that the rates of excretion of ammonia under the two sets of conditions differed greatly. In Experiments 2 and 3 there were not enough *Paramecia* to maintain a supply of food throughout the tests. After the food which was present had been exhausted, the rate of excretion decreased markedly. The curves for these experiments are similar to those obtained for *Paramecium* and *Spirostomum*, and they are to be interpreted in the same manner.

TABLE IV
RATE OF EXCRETION OF AMMONIA BY *Didinium**

Exp. No.	No. OF ANIMALS PER CC.	FOOD SUPPLY	PARTS OF AMMONIA BY WEIGHT PER 1,000,000 FOR 200 ANIMALS PER CC. AFTER—								
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.	9 Hr.
1.....	200	suf.	0.85	1.70		3.10			5.50		7.85
2.....	312	insf.	0.80	1.45		1.88		2.30			
3.....	250	insf.		1.18		2.00		2.00			
4.....	160	suf.	0.80	1.55		2.90		4.60			

* The amount of ammonia excreted by 200 animals per cubic centimeter of culture solution in 1-9 hours is given in parts by weight per 1,000,000 of solution. The abbreviations "suf." and "insf." denote respectively sufficient and insufficient food. Even in those experiments marked "insf." there were a few *Paramecia* present. Note that the concentration of ammonia in the culture containing much food increases from the beginning to the end of the experiment but that in the culture with little food it increases very little after 2 hours.

In Experiments 1 and 4, however, the food supply was adequate for the duration of the tests. Here the rate of excretion remained constant throughout the period of the experiments, and did not decrease as in Experiments 2 and 3. It is concluded from this direct comparison of the effects of starvation and satiety that the rate of excretion depends upon the presence or absence of a sufficient supply of food just as it does in man.

III. METHOD OF EXCRETION OF NITROGENOUS END-PRODUCTS OF METABOLISM IN PROTOZOA

It is often asserted that the nitrogenous end-products of metabolism in Protozoa are eliminated by the contractile vacuole. There is, however, considerable evidence which indicates that the vacuole is primarily concerned with the removal of excess water which ac-

cumulates in the cell. A summary of the evidence favoring each of these views follows:

Stein (1878) probably was the first to maintain that the function of the contractile vacuole is excretory. Stein and Schmidt (see Kent, 1880, p. 69) make the following statement: "The functions

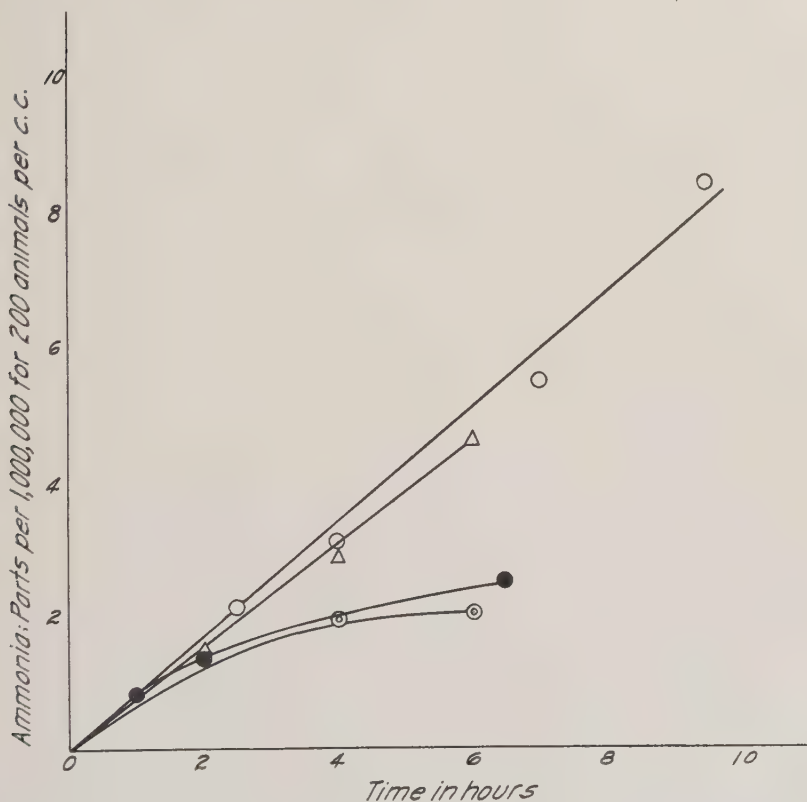


FIG. 4.—*Didinium*: amount of ammonia present at successive periods of time. ○, Exp. 1; ●, Exp. 2; ⊙, Exp. 3; △, Exp. 4.

discharged by the contractile vacuole are excretory, and correspond most nearly to those of the renal organs of the higher animals." Among others who have supported the excretory theory are Pritchard (1861), Griffiths (1888), Khainsky (1910), Woodruff (1911), Minchin (1912), and Howland (1924). The evidence given by Griffiths and contradicted by Howland has been presented above.

Khainsky (1910) found that an increase in temperature, within certain limits, is accompanied by a corresponding increase in the pulsation-rate. This was interpreted as indicating a higher rate of metabolism, the more rapid pulsation-rate being necessary to remove the increased amount of waste-products.

Experiments by Nowikoff (1908), Shumway (1917), the Riddle and Torrey (1923), in which the effects of thyroid feeding and the response of *Paramecia* to thyroxin were observed, offer further though indirect evidence in favor of the theory of excretion. Two definite effects were noted: a tendency toward the formation of extra vacuoles, and a marked acceleration in the rate of pulsation.

Flather (1919) found that adrenalin, pituitrin, and pineal gland extract produce similar results—a pronounced acceleration in the pulsation-rate, and a dilatation of the vacuoles. It is interesting to note in this connection that these drugs produce diuresis and dilatation in the vertebrate kidney, accompanying a general rise in blood-pressure; and that the diuresis and dilatation last longer than the increased blood-pressure, indicating a specific effect on the kidney itself.

In his discussion of the functions and homologies of the contractile vacuole in plants and animals Hartog (1888) suggested that it may be an organelle for regulating the hydrostatic pressure within the cell. This suggestion is based on the fact that in a system, such as a living cell presents, in which two fluids of different densities are separated by a semipermeable membrane, the less dense fluid is expected to pass through the membrane into the more dense fluid until a state of equilibrium is established. In the system formed by the cell, the endoplasm, the cell membrane, and the culture fluid represent respectively the more dense fluid, the semipermeable membrane, and the less dense fluid. If this obtains, endosmosis would be expected to take place, necessitating some form of mechanism to prevent excessive dilution of the endoplasm, and a hydrostatic pressure within the cell greater than the cell-membrane can withstand.

Zülzer (1910) found in experiments on *Amoeba verrucosa* that the pulsation-rate of the vacuole decreases with an increase in salt content of the culture-medium, and that concentrations of $1\frac{1}{2}$ – $2\frac{1}{2}$ per cent of salt cause the vacuole to cease pulsating, but that upon res-

toration of the animals to fresh water pulsation is resumed. This seems to indicate that at these concentrations of salt the exosmotic effect exerted by the culture medium is equal to or greater than the endosmotic effect exerted by the endoplasm. If this is true, there is no excess water within the cell to be removed, and the vacuole is functionless. Whether or not the cessation of pulsation in Zülzer's experiments was due to the isotonicity of the culture-medium with the endoplasm or to pathological conditions induced by the salt has not been ascertained.

Stempell (1914) constructed a mechanical system which shows clearly that osmosis can be made the causal agent for producing intermittent discharge of a fluid from a system. Doubtless this system was not intended to be compared with the contractile vacuole in any respect other than the pulsating effect.

The relation found by Khainsky (1910) between increases in temperature and increases in pulsation-frequency can be explained on the basis of the hydrostatic theory as follows. Van Rysselberghe (1902) found that the permeability of living membranes increases with an increase in temperature. If this is true, then an increase in temperature would necessitate the removal of a larger amount of water which passes through the membrane because of its increased permeability. If the function of the vacuole is the removal of the excess water, then an increase in temperature would necessitate a corresponding increase in pulsation-frequency. The observed increase in rate of pulsation therefore is not necessarily due to an increased rate of metabolism as Khainsky maintains.

In a previous paper the author (1927) presented further evidence bearing on the question as to whether or not the function of the contractile vacuole is primarily that of an excretory organelle. It was shown in this paper that urea cannot be detected in the fluid of the vacuoles of *Paramecium* by means of the xanthidrol precipitation test described by Fosse (1913). The xanthidrol reagent, which is sensitive to one part of urea in 12,000 by weight when used in this manner, was injected into the vacuole with the aid of suitable micro-injection apparatus. No trace of the characteristic needle-like crystals of di-xanthyl urea, which are precipitated by xanthidrol in the presence of urea, was found either in that part of the organism in

which the contractile vacuole is usually located or in the liquid surrounding the organism. It was consequently concluded that either urea is not present in the fluid of the vacuole or that its concentration is too low to be detected with the reagent used.

Further evidence bearing on the questions under consideration was obtained in experiments described below. These experiments consisted of removing the fluid from the vacuole and subjecting it to microchemical analysis.

MATERIAL AND METHODS

In these experiments *Spirostomum* was used. The organisms were washed free from culture fluid as previously described to prevent the possible introduction of error from contamination of the fluid of the vacuole by substances dissolved in the culture fluid.

The apparatus used for the removal of the contents of the contractile vacuole consisted of a Taylor (1925) pipette used in connection with a Chambers (1922) micromanipulator. The main body of the pipette was rigidly mounted on the table to the left of the micromanipulator and was connected to the pipette tip, which was mounted on the movable arm of the manipulator, by a glass tube approximately 40 cm. in length. This glass tube, having a diameter of 4 mm., was sufficiently flexible to permit a wide enough range of motion for the capillary tip. It is especially advantageous to have the main body of the pipette mounted separately and some distance removed from the capillary tip. This prevents to a considerable extent the transmission of vibrations from the pipette to the tip during adjustment and manipulation of the former. The organism was mounted on the lower surface of a cover glass. This cover glass formed the top of a rectangular glass chamber, the front of which was left open to allow the pipette entrance. The tip of the pipette was bent up at a right angle to facilitate its introduction into the body of the organism suspended on the lower surface of the cover glass.

By the use of this apparatus, fluid was removed from the vacuole and injected into a drop of a solution of urease hanging from the lower surface of another cover glass; then, after a short time, generally about 2 minutes, a drop of Nessler's reagent was added to the

drop of urease solution to ascertain whether or not ammonia was produced after the treatment of the contents of the vacuole with the enzyme.

RESULTS

Four experiments with *Spirostomum* such as described above were made. In two of these the contents of a single vacuole were removed; in the others the contents of two were removed. Upon the addition of Nessler's reagent the characteristic brown color produced by this reagent in the presence of ammonia was plainly visible under the microscope in all four experiments. In those in which the contents of two vacuoles were used the color developed was considerably deeper than in those in which the contents from only one were used. No indication of ammonia was obtained in other experiments in which the enzyme was not used. Nor did Nesslerization of a drop of urease solution alone show any trace of ammonia. The brown color obtained when the contents of the vacuole were added to the urease solution and then Nesslerized must therefore have arisen from ammonia produced by hydrolysis of urea dissolved in the fluid of the vacuole. It may consequently be concluded that urea is excreted by the contractile vacuole in *Spirostomum*. It will be recalled that the results obtained in experiments described in a preceding paper (Weatherby, 1927) indicate that this does not obtain in *Paramecium*. However, upon purely theoretical considerations, one would anticipate the fluid of the vacuole to contain dissolved in it at least a part of all soluble substances in the body. The fact, therefore, that urea was not found in *Paramecium* does not indicate that none was present; it merely indicates that the reagent used was not sensitive enough to give positive test at the concentration which obtained.

The question now arises as to whether the urea excreted by the contractile vacuole is sufficient in quantity to indicate that it is an excretory organelle. The depth of color developed in the tests for ammonia in the experiments on *Spirostomum* was, at most, comparable to that produced in a solution of ammonia containing one part by weight per 500,000. However, the fluid of the vacuole was diluted to from three to five times the original volume when added to the urease solution; this gives an original concentration of ap-

proximately one part by weight per 100,000 parts of fluid. Assuming that this was the concentration, what portion of the urea excreted by this form passes out through the contractile vacuole? As previously stated, the quantity of urea excreted by *Spirostomum* is approximately 1.25 parts by weight per 1,000,000 parts of culture fluid per hour for 500 animals per cubic centimeter. By ascertaining the rate of pulsation of the vacuole, and the volume of the vacuole, the quantity of fluid evacuated by a given number of organisms in a given length of time was computed. From this it was found that the concentration of urea in the fluid of the vacuole would be of the order of one part by weight per 1,000 parts of fluid, if all the urea excreted is eliminated through the vacuole. If the fluid of the vacuole, then, contains only one part of urea by weight per 100,000 parts of fluid, it is evident that not more than 1 per cent of the urea excreted passes through the vacuole.

On the basis of these considerations one is justified in concluding that the vacuole is not primarily an organelle of excretion as the term is usually defined. Moreover, the physical nature of the organism would lead one to predict that crystalloid substances such as urea, which are dissolved in the body fluid, readily pass directly to the exterior by dialysis. In all probability, then, this is the mechanism for the elimination of nitrogenous end-products of metabolism.

SUMMARY

1. The bulk of the nitrogen excreted by *Paramecium* and *Spirostomum* is in the form of urea. No excretion of ammonia, uric acid, creatin, or creatinin was found. The bulk of the nitrogen excreted by *Didinium* is in the form of ammonia. In experiments lasting for 24 hours or more a trace of uric acid was sometimes found, but no urea, creatin, or creatinin.

2. In *Paramecium* the initial rate of excretion of urea is approximately 1.40 parts by weight per 1,000,000 per hour for 2,500 animals per cubic centimeter.

3. In *Spirostomum* the initial rate of excretion of urea is approximately 1.30 parts by weight per 1,000,000 per hour for 500 animals per cubic centimeter.

4. In *Didinium* the rate of excretion of ammonia is approximately 0.80 parts by weight per 1,000,000 per hour for 200 animals per cubic centimeter. The rate of excretion of uric acid in this form is not sufficient to permit measurements with any considerable degree of accuracy.

5. Substances frequently used in the preparation of culture media—hay, wheat, barley, rye, rolled oats, malted milk, beef extract, blood fibrin, and blood albumen—contain considerable quantities of uric acid. This probably accounts for the fact that Howland found uric acid in cultures of Protozoa.

6. Methods for quantitative estimation of the amount of urea in the fluid of the contractile vacuole were developed. In *Spirostomum* this was found to be approximately one part by weight per 1,000,000.

7. The amount of urea which should be in the fluid of the vacuole, if all the urea excreted by this form is eliminated through the vacuole, is approximately one part by weight per 1,000, or about 100 times the amount which was actually found. In *Spirostomum*, then, the contractile vacuole does not function primarily as an excretory organelle, as the term is generally used.

8. That urea was not detected in the fluid of the vacuole in *Paramecium* in previous experiments was no doubt due to the fact that the reagent used was not sensitive enough to give a positive indication at the dilution which actually obtains. The reagent in these experiments was sensitive to only one part of urea by weight per 12,000. The actual concentration of urea in the fluid of the vacuole in *Paramecium*, if *Paramecium* may be compared to *Spirostomum* in this respect, is more nearly of the order of one-tenth that amount.

9. The physical structure of a protozoan such as *Paramecium* or *Spirostomum* indicates that at least part of all soluble substances contained within the body pass to the exterior through the cell membrane by the process of dialysis. If this obtains, a part of the urea, which is an excretory product in these forms, is excreted through the vacuole; but the greater part passes by dialysis directly to the exterior through the cell membrane.

LITERATURE CITED

- ADOLPH, E. F. 1922. The physiological action of excretory products. *Anat. Rec.*, XXIV, 396.
- CHAMBERS, R. 1922. New apparatus for the dissection and injection of living cells. *Ibid.*, pp. 1-19.
- FLATHER, M. D. 1919. The influence of the glandular extracts upon the contractile vacuoles of *Paramecium caudatum*. *Biol. Bull.*, XXXVII, 22-39.
- FOSSE, R. 1913. Sur l'identification de l'urée et sa précipitation de solution extrêmement diluées. *Comp. Rend.*, CXXXVII, 948-57.
- GRIFFITHS, A. B. 1888. A Method of demonstrating the presence of uric acid in the contractile vacuoles of some of the lower organisms. *Proc. Royal Soc. Edin.*, XVI, 131-35.
- HARTOG, M. M. 1888. Preliminary notes on the functions and homologies of the contractile vacuole in plants and animals. *Rep. British Assoc. Adv. Sci.*, pp. 714-16.
- HOWLAND, RUTH B. 1924. On excretion of nitrogenous waste as a function of the contractile vacuole. *Jour. Exp. Zool.*, XL, 231-50.
- KENT, W. S. 1880. A manual of the infusoria. London. Pp. 913.
- KHAINSKY, A. 1910. Zur Morphologie und Physiologie einiger Infusorien (*Paramecium caudatum*) auf Grund einer neuen histologischen Methode. *Arch. f. Prot.*, XVI, 1-60.
- MINCHIN, E. A. 1912. An introduction to the study of the protozoa. London. Pp. 504.
- MORSE, W. 1927. Applied biochemistry. Philadelphia and London. Pp. 988.
- NOWIKOFF, M. 1908. Über die Wirkung des Schilddrüsen Extrakts und einiger anderer Organstoffe auf Ciliaten. *Arch. f. Prot.*, XI-XII, 309-26.
- PRITCHARD, A. 1861. A history of the infusoria. London. Pp. 986.
- RIDDLE, M. C., and TORREY, H. B. 1923. The physiological response of *Paramecium* to thyroxin. *Anat. Rec.*, XXIV, 396.
- SHUMWAY, WALDO. 1917. The Effect of a thyroid diet on *Paramecium*. *Jour. Exp. Zool.*, XXII, 529-62.
- STEIN, F. 1878. Der Organismus der Infusionsthier nach eigenen Forschungen in systematischer Reihenfolge bearbeitet. Leipzig. Div. 3, p. 154.
- STEMPELL, W. 1914. Über die Funktion der pulsierenden Vacuole und einen Apparat zur Demonstration derselben. *Zool. Jahrb., Abt. f. Allg. Zool. und Physiol. d. Thiere*, XXXIV, 437-78.
- TAYLOR, C. V. 1925. Improved micromanipulation apparatus. *Univ. Cal. Pub. Zool.*, XXVI, 443-54.
- VAN RYSELBERGHE, Fr. 1902. Influence de la température sur la perméabilité du protoplasme vivant pour l'eau et les substances dissoutes, *Rec. Inst. Bot. Brux.*, V, 209-49.
- ZÜLZER, MARGARET. 1910. Über den Einfluss des Meerwassers auf die pulsierende Vacuole. *Arch. f. Entw. d. Org.*, XXIX, 632-40.

VITA

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In September, 1924, he enrolled in the Johns Hopkins University as a graduate student in the Department of Zoölogy, from which he received the degree of Master of Arts in 1926. The following year—1926-1927—was spent in the employ of the U.S. Bureau of Fisheries. In September, 1927, he re-entered the Johns Hopkins University, and completed the requirements for the degree of Doctor of Philosophy in June of the following year.

